CATTLE BETA-CASEIN GENE TARGETING VECTOR USING HOMOLOGOUS RECOMBINATION

Technical Field

5   The present invention relates to bovine beta-casein gene targeting vectors that function through homologous recombination, bovine somatic cells that are gene-targeted with the said vectors and embryos that are nuclear-transferred with the said bovine somatic cells. The present

10   invention also relates to methods for producing amounts of desired proteins from the milk of the transgenic cattle that are prepared by implanting the said embryos.

Background Art

15   Our continuous efforts in animal genomics to identify genes and their functions have made it possible to generate transgenic animals. Transgenic animals have been created great commercial value in industries such as biomedicine and agriculture. Various techniques such as microinjection,

20   viral transfection, sperm vector, application of embryonic stem(ES) cells and somatic cell nuclear transfer(SNCT) have also been developed to prepare transgenic animals or so called genetically-modified animals.

       The microinjection method, which injects a DNA

25   molecule into the male or female pronucleus of fertilized eggs (Harbers et al., Nature., 293(5833); 540-2, 1981; Brinster et al., Cell., 27;223-231, 1981; Gordon et al., Proc Natl Acad Sci USA., 77(12);7380-7384; Costantini et al., Nature., 294(5836);92-94), has been widely employed to

30   produce transgenic animals (Hammer et al., Nature.,

315(6021);680-683, 1985; van Berkel et al., Nat Biotechnol.,
20(5);484-487, 2002; Damak et al., Biotechnology(NY),
14(2);185-186, 1996). However, the efficiency of this
technique in the production of transgenic animals is very

5   low in that only 2-3% of the injected eggs give rise to
transgenic offspring (Clark et al., Transgenic Res., 9;263-
275, 2000). The production of transgenic animals using the
microinjection method is also a labor-intensive and costly
procedure requiring large numbers of animals and facilities

10   (Brink et al., Theriogenology, 53;139-148, 2000). Another
disadvantage of this technique is that it cannot control
the integration sites and the copy numbers of the inserted
genes. The resulting random integration sometimes leads to
a low or nonspecific expression of the transgenes. It was

15   further reported that the unregulated expression of certain
transgenes sometimes cause lethality in the embryonic
development (Wei et al., Annu Rev Pharmacol Toxicol.,
37;119-141, 1997).

The retrovirus-mediated method is also widely used in

20   order to genetically manipulate animals (Soriano et al.,
Genes Dev., 1(4);366-375, 1987; Hirata et al., Cloning Stem
Cells., 6(1);31-36, 2004). Under this technique, the
desired gene sequences are introduced into the animal
genomes by using virus-mediated vectors. Although viral

25   transformation is more efficient than pronuclear injection,
random insertion of the foreign genes and mosaicism are
entailed due to multiple integrations (Piedrahita et al.;
Theriogenology, 53(1);105-116, 2000). Additionally, the
maximum size of the introduced genes is usually limited to

30   approximately 7 kb and there is concerning of the potential

2

interference caused by virally encoded proteins (Wei et al., Annu Rev Pharmacol Toxicol., 37;119-141, 1997; Yanez et al., Gene Ther., 5(2);149-159, 1998).

To circumvent the problems referred to above, a gene targeting technique that can insert or remove a DNA segment at a specific location was developed. The gene targeting technique was first applied to the mouse embryonic stem cells to study gene function. Mouse embryonic stem cells are now being used to introduce predetermined genetic modifications into embryos. A number of specific gene-targeted mice have been produced through the manipulation of mouse embryonic stem cells using the technique (Brandon et al., Curr Biol., 5(6);625-634, 1995; Capecchi et al., Science, 244(4910);1288-1292, 1989; Thompson et al., Cell, 56(2);313-321, 1989; Hamanaka et al., Hum Mol Genet., 9(3);353-361, 2000; Thomas et al, Cell, 51(3);503-512, 1987; te Riele et al., Proc Natl Acad Sci USA, 89(11);5182-5132, 1992; Mansour et al., Nature, 336(6197), 348-352, 1988; Luo et al., Oncogene, 20(3);320-328, 2001). The extension of this gene targeting method to other mammalian species, particularly livestock, could bring numerous biomedical benefits such as mass production of pharmaceutical proteins and animal disease models.

Until now, most recombinant therapeutic proteins have been produced by cell culture systems, which use cells such as yeast, bacteria or animal cells. However, it is difficult to produce proteins in large scale using cell culture systems because of the limited capacity and high cost. Furthermore, for some proteins, additional steps are

required to introduce proper posttranslational modifications such as glycosylation, γ-carboxylation, hydroxylation and so on (Houdebine et al., Transgenic Res., 9(4-5);305-320, 2000; Lubo et al., Transgenic Res., 9(4-5);301-304, 2000).

Animal bioreactors that produce valuable or therapeutic proteins have been evaluated as efficient and cost-effective expression systems. In particular, the large-scale production of therapeutic recombinant proteins from transgenic animals is much more cost-effective compared to the cell culture system (van Berkel et al., Nat Biotechnol., 20(5);484-487, 2002). Recombinant proteins produced in animal milk were known to be post-translationally modified in a way very similar to the human counterpart proteins (Edmunds et al., Blood, 91(12);4561-4571, 1998; Velander et al., Proc Natl Acad Sci USA., 89(24);12003-12007, 1992; van Berkel et al, Nat Biotechnol., 20(5);484-487, 2002).

Cow's milk is composed of approximately 88% water, 3.3% protein and the remaining carbohydrates and fat. The caseins, comprising 80% milk protein, are divided into four groups, alpha S1, alpha S2, beta and kappa casein. Beta casein is the most abundant protein in milk and is expressed in a concentration of 10mg/ml in bovine milk (Brophy et al., Nat Biotechnology., 21(2);157-162, 2003).

The somatic cell nuclear transfer (SCNT) technique is more efficient way to make transgenic animals compared to the microinjection method because almost all of the cloned animals derived from transformed somatic cells are

transgenic animals (Brink et al., Theriogenology, 53;139-148, 2000). It is also possible to predetermine the sex of animals and create a genetically homogeneous herd in order to produce a uniform product (Lubo et al., Transgenic Res.,

5   9(4-5);301-304, 2000; van Berkel et al., Nat Biotechnol., 20(5);484- 487, 2002).

      Until now, ES cells and vector constructs for targeting a specific gene have been considered as

10   prerequisite elements to generate gene-targeted animals. However, there is a limitation in the use of ES cells from large livestocks, although some studies have developed ES-like cells in pig and cattle (Doetschman et al., Dev Biol., 127(1);224-227, 1988; Stice et al., Biol Reprod.,

15   54(1);100-110, 1996; Sukoyan et al., Mol Reprod Dev., 36(2);148-158, 1993; Iannaccone et al., Dev Biol., 163(1);288-292, 1994; Pain et al., Development, 122(8);2339-2348, 1996; Thomson et al., Proc Natl Acad Sci USA., 92(17);7844-7848, 1995; Wheeler et al., Reprod Fertil

20   Dev., 6(5);563-568, 1994).

      Instead, the use of normal somatic cells as nuclear donor cells has been suggested as an efficient and practical method to produce transgenic cattle (Brophy et al., Nat Biotechnol., 21(2);157-162, 2003; Cibelli et al.,

25   Science, 280(5367);1256-1258, 1998; Campbell et al., Nature, 380(6569);64-66, 1996; Wilmut et al., Experientia, 47(9);905-912, 1997; Denning et al., Cloning stem cells, 3(4);221-231, 2001), suggesting the possibility that somatic cells instead of embryonic stem cells can be used

30   for targeting specific genes.

With the application of SCNT technique, promoter
regions of milk protein genes have been used to direct the
expression of recombinant protein in the milk of transgenic
5   large animals (Schnieke et al. Science, 278(5346);2130-2133,
1997; Baguisi et al., Nat Biotechnol., 17(5);456-461, 1999;
Brophy et al., Nat Biotechnol., 21(2);157-162, 2003).
However, the use of this technique in farm animals is still
not practical unless the problems of low expression and/or
10 ectopic expression due to the random insertion of genes are
solved.   The ectopic expression of foreign protein causes
early embryonic lethality and is particularly severe in
nervous system, as most nervous system structures develop
in late embryonic and early postnatal stages (Gao et al.,
15 Neurochem Res., 24(9);1181-1188, 1999).   To eliminate or
reduce these side-effects, a new method that allows the
foreign protein to be expressed only during the lactation
period and strictly in the mammary gland have been invented
(Houdebine et al., Transgenic Res., 9(4-5);305-320, 2000).
20 Gene-targeting, known as the introduction of site-specific
modification into a genome by homologous recombination
event, is a powerful tool for tissue-specific expression of
recombinant proteins (Muller et al., Mech Dev., 82(1-2);3-
21, 1999; Clark et al., J Mammary Gland Biol Neoplasia.,
25 3(3);337-350, 1998).

The generation of a first knock-in ovine has opened
the door to make it possible to produce therapeutic foreign
protein-targeted large animals (McCreath et al., Nature,
30 405(6790);1066-1069, 2000).   The COLT-2 targeting vector

6

which has homology regions for COL1A1 gene that is highly expressed in fibroblasts was developed, allowing the promoter-trap enrichment of gene-targeting events. AATC2 trasgene comprised of human α1-antitrypsin (AAT)

5   complementary DNA within an ovine beta-lactoglobulin (BLG) expression vector was designed to direct expression in the mammary gland, having separate transcription unit. The amount of AAT secreted from targeted lambs was 37-fold more than that from the sheep with multiple and random

10   integration of the genes (McCreath et al., Nature, 405(6790);1066-1069, 2000). Thus, gene targeting is now regarded to be the most powerful method to produce large amount of therapeutic protein. However, the application of the promoter-trap targeting vector is limited to

15   transcriptionally active genes in the somatic cells. In general, transcriptionally active genes are more amendable to gene targeting than silent genes because they have a higher frequency of homologous recombination (Kuroiwa et al., Nat Genet., 36(7);775-780, 2004).

20

Milk proteins are expressed in a tissue-specific manner in the mammary gland. An over-expression of foreign protein is possible without causing lethality in embryonic or post-natal development by manipulating a gene coding of

25   the milk proteins. Among such proteins, beta-casein would be one of the best candidates since it is expressed abundantly. The bovine beta-casein exists as a single copy gene in total genome and is not known to be expressed in the somatic cells except for those in the mammary gland.

30   The targeting of foreign genes into the beta-casein gene,

7

and intron 5, 6 and 7 of bovine beta-casein gene; wherein the nucleic acid segment corresponding to the first region is located upstream to the nucleic acid segment corresponding to the second region in the 5'-3' arrangement

5   of beta-casein gene.

It is another object of the present invention to provide a bovine somatic cell which is bovine beta-casein gene-targeted with the said targeting vector.

It is another object of the present invention to

10 provide an embryo which is nuclear-transferred with the said somatic cell.

It is another object of the present invention to provide a method for preparing bovine beta-casein gene-targeted somatic cell, which comprises the steps of (1)

15 introducing the above vector into a bovine somatic cell; (2) occurring homologous recombination events in the bovine somatic cell; and (3) selecting the bovine beta-casein gene-targeted somatic cell.

It is another object of the present invention to

20 provide a method for preparing transgenic cattle, which comprises the steps of (1) introducing the above vector into a bovine somatic cell; (2) occurring homologous recombination events in the bovine somatic cell; (3) selecting the bovine beta-casein gene-targeted somatic

25 cell; (4) introducing the above gene-targeted cell into a nuclear-removed bovine oocyte to produce nuclear-transferred embryo; and (5) implanting the above embryo into a surrogate to produce cloned transgenic cattle.

It is yet another object of the present invention to

30 provide a method for preparing a large scale of desired

9

which is not expressed in the normal somatic cells, cannot be carried out by vectors utilizing the promoter trap.

5   Based on this background, the present inventors have created targeting vector cassettes specific for the bovine beta-casein gene, vectors inserted with a foreign gene using the said cassettes, bovine somatic cell introduced with the said vector, and nuclear-transferred embryo with the said bovine cell. The present inventors found that the

10   foreign gene was correctly targeted to the beta-casein gene of bovine genomic DNA and confirmed the targeting events with the said vector were highly efficient. Therefore, the present inventors have been completed transgenic cattle which could produce a large scale of desired therapeutic

15   protein using the said bovine beta-casein gene targeting vector cassette.

Summary of the Invention

20   It is an object of the present invention to provide a bovine beta-casein gene targeting vector comprising (1) a first region having a length of 5 to 12 kb which is homologous to the promoter and its flanking nucleic acid sequences of bovine beta-casein gene, and comprising exon 1,

25   intron 1, and exon 2 of bovine beta-casein gene; (2) a region for cloning a nucleic acid coding for desired proteins; (3) a region for coding a positive selection marker; (4) a second region having a length of 2.8 to 3.5 kb which is homologous to the nucleic acid sequences of

30   bovine beta-casein gene, and comprising exon 5, 6, 7 and 8,

8

therapeutic proteins from milk of cloned cattle produced
using the above method.

Brief Description of Drawings

5

FIG. 1 depicts 18.8kb pBCKI vector cassette targeting
the bovine beta-casein gene, derived from a pBluescript II
SK(+) plasmid. The vector also includes Sac II, Not I, and
BamH I restriction sites in front of *neo* gene and BamH I site
10 at the back of short arm region.

FIG. 2 depicts 14.8 kb pBCKI II vector cassette
targeting the bovine beta-casein gene, derived from a pGEM-
7Zf(+) plasmid (Promega).

15

FIG. 3 depicts 16.1kb pBCKIDT II vector cassette
targeting the bovine beta-casein gene, derived from pGEM-
7Zf(+) plasmid. The vector also includes Sac II, Not I, and
BamH l restriction sites in front of *neo* gene and BamH I site
20 at the back of short arm region. In addition, 1.3 kb
diphtheria toxin (DT) gene as a negative selection marker
is inserted.

FIG. 4 shows homologous recombination between pBCKI I
25 vector and beta-casein gene in genome. Double crossover
results in the replacement of the endogenous beta-casein
gene with the sequences in the vector cassette.

FIG. 5 shows homologous recombination between pBCKI II
30 vector and a beta-casein gene in genome. A partial

10

sequence of a beta casein gene locus in genome is replaced
with a partial sequence in a vector cassette as result of a
double crossover event.

5   FIG. 6 shows homologous recombination between
pBCKIDT II vector and a beta-casein gene in genome. Double
crossover results in the replacement of the endogenous
beta-casein gene with the sequences in the vector cassette.
As results of homologous recombination events, DT gene is
10   deleted.

         FIG. 7 shows PCR screening strategy used to identify
gene targeting events at the bovine beta-casein locus and
the DNA sequences spanning the junctions of the targeted
15   beta-casein locus and the restriction sites in the
targeting vector cassettes, pBCKI I and pBCKI II. The upper
portion shows the approximate position of two primers used
to amplify the 4 kb fragment, from the neo gene region
unique to the beta-casein gene targeting vectors to the
20   endogenous beta-casein locus which is not used in the
vector cassettes. Sequence A shows a portion of DNA
sequence spanning the junction between the long arm region
and the neo gene. Sequence B shows a portion of DNA
sequence spanning the junction between the neo gene and the
25   short arm region. The position and orientation of the 5'
PCR primer is shown. Sequence C shows a portion of DNA
sequence spanning the junction between the short arm region
and the endogenous beta-casein gene locus which is excluded
from the BCKI I and BCKI II vectors.   The position and
30   orientation of the 3' PCR primer is shown.

FIG. 8 shows PCR screening strategy used to identify gene targeting events at the bovine beta-casein locus and the DNA sequences spanning the junctions of the targeted
5   beta-casein locus and the restriction sites in the targeting vector cassette, pBCKIDTⅡ. The upper portion shows the approximate position of two primer sets used to amplify the 4 and 3.4 kb fragments, from the *neo* gene region unique to the beta-casein gene targeting vectors to
10   the region endogenous beta-casein locus which is not used in the vector cassettes. Here, after amplifying 4 kb PCR fragments using the first primer set, 3.4 kb PCR fragments were amplified using the second primer set for a small volume of DNA samples. Sequence A shows a portion of DNA
15   sequence spanning the junction between the long arm region and the *neo* gene. Sequence B shows a portion of DNA sequence spanning the junction between the *neo* gene and the short arm region. The positions and orientations of the 5' PCR primers are shown. Sequence C shows a portion of DNA
20   sequence spanning the junction between the short arm region and the endogenous beta-casein gene locus which is excluded from the BCKI Ⅰ and BCKI Ⅱ vectors. The positions and orientations of the 3' PCR primers are shown.

25   FIG. 9 shows the pBC10 vector constructed in our previous study (Kim et a l., J Biochem (Tokyo)., 126(2);320-5, 1999). The pBC10 vector is based on a pBluescriptⅡ SK vector backbone and includes the bovine beta-casein promoter region, which is 10kb in length. The
30   promoter region contains 8 kb of the 5'-flanking sequence

of gene, the untranslated exon 1 and 2 (vertical open boxes), and 2 kb of intron 1 of bovine beta-casein gene. The promoter has restriction enzyme sites for Sac I , Aat II , and Sac II , but there is no recognition sequences of other
5 restriction enzymes such as Sma I , BamH I , Sal I , Spe I and Cla I.

     FIG. 10 shows the isolated 10 kb DNA fragment from the pBC10 vector digested with Sac I and Sac II restriction
10 enzymes. The isolated 10 kb DNA fragment is used for a long arm of the pBCKI I vector cassette.

     FIG. 11 shows the isolated 6 kb DNA fragment from the pBC10 vector digested with Aat II and Sac II restriction
15 enzymes. The isolated 6 kb DNA fragment is used for a long arm of the pBCKI II vector cassette.

     FIG. 12 shows construction procedures of the pBC3.1 vector applied for the short arm of pBCKI I and pBCKI II
20 vector cassettes. The 3.2 kb DNA fragment including exon 5, 6, 7 and 8 of bovine beta-casein gene was prepared by PCR amplification using the primer set which has Xho I and Sal I sites (bold characters) from the bovine chromosomal DNA. The amplified PCR fragment was digested with Xho I and Sal I
25 restriction enzymes and then ligated into the Sal I restriction enzyme site of pGEM-T vector (Promega). The 3.2 kb DNA fragment in the pGEM-T vector was digested with Hinc II and Sal I restriction enzymes and then ligated into Sal I site of pBluescript II SK(+) vector.

30

FIG. 13 shows the procedure obtaining the *neo* gene including the SV40 *ori*, the early promoter, the neomycin resistance gene, the SV40 early splicing region and polyadenylation site from a pMAM*neo* vector (CLONTECH). The

5   2.7 kb *neo* gene fragment digested with BamH I restriction enzyme was ligated into BamH I site of pBluescript II SK(+) vector. The 2 kb DNA fragment of the *neo* gene in a pBluescript II SK(+) vector was digested with Bgl II and BamH I restrction enzymes and ligated into Bgl II site of pSP73

10 vector (Promega). The 2 kb fragment of a pSP73 vector digested with Bgl II and EcoR V restriction enzymes was then re-ligated into Bgl II and EcoR V sites of the pBluescript II SK(+) vector containing the 0.7 kb *neo* gene to generate the pneo2.7 vector construct.

15

FIG. 14 shows the construction procedures of the pBCKI I and pBCKI II vector cassettes. The DNA fragments corresponding to a positive selection marker and short arm in pBluescript II SK(+) were digested with respective

20 restriction enzymes, and then ligated into the BamH I , EcoR V , and Sal I site of pBluescript II SK(+) vector harboring pBC10 DNA fragment. The resultant pBCKI I vector includes the 10 kb long arm, the selection marker gene and the short arm. To shorten long arm length of the pBCKI I ,

25 the DNA fragment digested with Aat II and Sal I restriction enzymes was ligated into Aat II and Xho I sites of pGEM7Zf(+) vector. The resultant pBCKI II vector includes the 6 kb long arm, the *neo* gene, and the short arm.

30   FIG. 15 shows the procedure obtaining the DT gene,

inserted into pBCKIDT II vector, from pKO SelectDT (Lexicon Genetics). Using restriction enzyme, Rsr II, diphtheria toxin A (DT) gene containing SV40 Poly A and RNA polymerase II promoter was isolated. After klenow-filling

5   the isolated DT gene, it was inserted into Hind II sites of pBluescript II SK(+) vector (Stratagene), generating pSK DT vector. The DT gene in the pSKDT vector was digested with Xho I and EcoR V restriction enzymes and ligated into Xho I and Pvu II sites of a pSP73 vector (Promega) to generate the

10 pSP73DT vector construct.

    FIG. 16 shows the construction procedures of the pBCKIDT II vector cassette. DT gene in pSP73DT vector was digested with restriction enzyme, Xho I and Sal I and then

15 ligated into Sal I site of pBCKI I vector cassette, generating pBCKIDT I vector. The resultant pBCKIDT I vector was digested with Aat II and Sal I and then ligated into Aat II and Xho I sites of pGEM-7Zf(+) vector (Promega), generating pBCKIDT II vector cassettes.

20

    FIG. 17 shows introduction of a valuable gene into the pBCKI I vector. As an example of the genes for therapeutic proteins, human thrombopoietin (hTPO) cDNA was inserted into the invented pBCKI I vector cassettes. The

25 1.0 kb of the hTPO cDNA coding for the full length form was amplified by PCR in our previous study, and the poly(A) additional signal sequence of the bovine growth hormone (bGH) gene, which is 300bp in length, was ligated into the Kpn I site to downstream of the hTPO cDNA fragment (Sohn, DNA

30 Cell Biol., 18(11);845-852, 1999). The 1.3 kb DNA fragment

15

including hTPO cDNA and bGH gene was inserted into Sac II and Not I sites of the pBCKI I vectors, generating the 20.1 kb pBCTPOKI I vector construct.

5   FIG. 18 shows introduction of the hTPO cDNA into pBCKI II vector cassette. The resultant 16.1 kb pBCTPOKI II construct was generated and the procedures were the same as described in FIG. 17.

10   FIG. 19 shows introduction of the hTPO cDNA into pBCKIDT II vector cassette. As a result, 17.4 kb pBCTPOKIDT II construct was generated and the construction procedures were the same as described in FIG. 17.

15   FIG. 20 shows the linearized pBCTPOKI I vector to be used for transfection after Sal I digestion.

        FIG. 21 shows the linearized pBCTPOKI II vector to be used for transfection after Aat II digestion.

20

        FIG. 22 shows the linearized pBCTPOKIDT II vector to be used for transfection after deleting a plasmid vector by Aat II and Cla I digestion.

25   FIG. 23 shows the linearized pBCTPOKI II vector to be used for transfection after deleting a plasmid vector by Aat II and Cla I digestion. Whereas the pBCTPOKI II vector, described in FIG. 21, was simply linearized and transfected into cells with a plasmid vector, the pBCTPOKI II vector, 30 described here, was transfected into cells after deleting a

plasmid vector by two restriction enzyme digestion.

FIG. 24 is photographs showing morphologies of bovine
embryonic fibroblasts (bEF) and bovine ear skin fibroblasts
5 (bESF). A and B are non-transfected (normal) cells, bEF
and bESF, respectively, cultured to confluence. C and D
show the colony formed after transfection of the targeting
vector into bEF and bESF, respectively. After the
targeting vector which has antibiotic resistance gene, neo
10 gene, is introduced into bEF and bESF using the
Lipofectamine™ 2000 reagent (Invitrogen) method, the
transfected cells were treated with G418 (Gibco, Invitrogen
corporation) and then only the survived colonies were
subject to analyses of the inserted foreign genes.
15

FIG. 25 shows PCR analysis of the survived colonies
after pBCKI II transfection. The positive signals for the
500 bp PCR fragment indicated by an arrow represent
transfected cell clones, were denoted as "+" and the
20 negative signals were denoted as "-". The pBCTPOKI II vector
of the present invention was used as a positive control and
the PCR was amplified with the primers specific for hTPO
gene.

25   FIG. 26 and FIG. 27 depict construction procedures of
the pneoBC3.7 vector to be used as the control for long-
range PCR analysis. The 591bp DNA fragment harboring intron
8 and exon 9 corresponding to bases 7888 to 8479 of bovine
beta-casein gene was prepared separately by PCR
30 amplification using a primer set from the bovine

chromosomal DNA. The 3' primer has Xho I site depicted as bold characters. The amplified PCR fragment was ligated into pGEM-T vector to generate the pBC591 vector construct. The pBC591 vector was digested with Sal I and Xho I

5   restriction enzymes, and then the resultant DNA fragment was ligated into Sal I site of the pBC3.1 vector. At the same time, the pneo2.7 vector digested with BamH I and EcoR V restriction enzymes, and then the resultant DNA fragment was ligated into the pBC3.1 vector digested with BamH I and

10   EcoR V restriction enzymes to generate the pneoBC3.7 vector. The arrows show primer set for 4 kb long-range PCR analysis.

FIG. 28 shows the results of long-range PCR analyses of the bESF cell colonies transfected with pBCTPOKI II The

15   positive signals for the 4 kb indicated by arrows represent targeted cell clones, were detected in two colonies, "81" and "89", and the other colonies showed negative signals (-). The pneoBC3.7 vector was used as a positive control.

20   FIG. 29 shows the results of long-range PCR analyses of the bESF cell colonies transfected with pBCTPOKI II and pBCTPOKIDT II. The positive signals for the 3.4 kb indicated by arrows represent targeted cell clones. The first PCR analysis was performed from approximately five cells and

25   then the second PCR analysis was performed from the first PCR products, showing resultant 3.4 kb positive signals (FIG. 8). A is the results of long-range PCR analyses of cell colonies transfected with pBCTPOKIDT II vector and the number 5, 30, 17, 18, 20, 21, and 26 cell colonies were

30   identified to be gene-targeted. B is the results of long-

range PCR analyses of cell colonies transfected with
pBCTPOKI Ⅱ and the number 16 cell colony was identified to
be gene-targeted. C is the results of long-range PCR
analyses of wild-type bovine genomic DNA used as a negative
5   control (-) and pneoBC3.7 vector used as a positive control
(+).

FIG. 30 shows strategy for Southern blot analysis to
identify the targeted nucleic acid sequences. The BCTPOKI Ⅱ
10   vector and purified genomic DNAs from transfected cell
clones were digested with EcoRI. The size of DNA fragments
from BCTPOKI Ⅱ vector (A) and genomic DNA (B) digested with
the EcoR I restriction enzyme are 9.2 kb and 9.9 kb,
respectively. The bar under the hTPO gene represents the
15   locus of the probe used for Southern analysis. 500 bp
fragments of hTPO cDNA used for the probe were amplified by
PCR.

FIG. 31 shows identification of targeted colonies by
20 . Southern blot analysis. Two colonies, 81 and 89, were
identified to be targeted at the endogenous bovine beta-
casein gene locus with the pBCTPOKI Ⅱ vector of the present
invention. The targeted colonies represented the 9.9 kb
fragment and the other colonies, 97, 47, 43 and 34, did not
25   show any signal. The bovine genomic DNA was used at the
various concentrations as a negative control and the 9.2kb
fragments derived from the pBCTPOKI Ⅱ vector on various
concentrations were used as a positive control.

30   FIG. 32 shows PCR analysis of nuclear-transferred

19

embryos from 81 cell clone. The positive (+) signals, 356 bp in length, implied that the cloned embryos derived from the somatic cells that were targeted with the pBCTPOKI II vector of the present invention. The pBCTPOKI II vector was

5   used as a positive control. And nested PCR was also performed to re-confirm the positive signals.

FIG. 33 is a photograph showing a 89 cell clone derived fetus enclosed with a fetal membrane collected at

10   36 days of gestation (A), the fetal membrane-removed fetus (B) and cells derived from the fetal membrane (C), the result of the long-range PCR of cells derived from the fetal membrane (D). The positive signals for the 4 kb indicate that cells, genetically identical to the cloned

15   fetus, were targeted with the pBCTPOKI II vector of the present invention. The pneoBC3.7 vector was used as a positive control.

Detailed Description of the Invention

20

The present invention relates to preparing bovine beta-casein gene targeting vectors used to generate beta-casein gene-targeted cattle which produce amounts of desired therapeutic proteins from milk. Whereas goat and

25   sheep produce milk to 300 and 200 liters, respectively, a year, cattle produce milk up to 6,300 liter a year. Cattle are optimal species that could be used as animal bioreactors, however, there has been no report about targeting the desired gene to milk protein genes in bovine.

30   The present invention relates to vector constructs which

can target to a bovine beta-casein gene of bovine milk protein genes and a method preparing beta-casein gene targeted cattle which produce amounts of therapeutic proteins using the vector constructs of the present
5   invention.

In an aspect, the present invention relates to a bovine beta-casein gene targeting vector comprising (1) a first region having a length of 5 to 12 kb which is
10 homologous to the promoter and its flanking nucleic acid sequences of bovine beta-casein gene, and comprising exon 1, intron 1, and exon 2 of bovine beta-casein gene; (2) a region for cloning a nucleic acid coding for desired proteins; (3) a region for coding a positive selection
15 marker; (4) a second region having a length of 2.8 to 3.5 kb which is homologous to the nucleic acid sequences of bovine beta-casein gene, and comprising exon5, 6, 7 and 8, and intron 5, 6 and 7 of bovine beta-casein gene; wherein the nucleic acid segment corresponding to the first region
20 is located upstream to the nucleic acid segment corresponding to the second region in the 5'-3' arrangement of beta-casein gene.

The term "gene targeting vector", as used herein, means a vector that can remove or insert a desired gene to
25 a specific-genomic locus and includes a nucleic acid sequence homologous to a particular gene for homologous recombination. A gene targeting vector of the present invention is a beta-casein targeting vector which inserts nucleic acid sequences coding desired proteins to beta-
30 casein gene in genome in the 5'-3'arrangement. The terms,

vectors   and   vector   cassettes   are   used   herewith
interchangeably, and the vectors can be either circular or
linear form. The gene-targeting vectors of the present
invention are bovine beta-casein gene-targeting vectors.

5   The   beta-casein   targeting   vector   of   the   present
invention comprises a first region and a second region that
are homologous to the sequences of the beta-casein gene
that are located before and after the cloning site.   A
first   region   corresponds   to   the   long   arm   and   a   second

10   region corresponds to the short arm.

Among   components   of   the   invented   bovine   beta-casein
gene targeting vectors, particularly, "a first region" and
"a second region" are important parameters to determine the
gene targeting efficiency.

15   "A   first   region"   of   herein,   is   characterized   by
having   a   length   of   5   to   12kb   which   has   a   DNA   sequences
homologous to the promoter and its flanking sequences of
bovine beta-casein gene, and comprising exon 1-2 and intron
1 of bovine beta-casein gene.   A bovine beta-casein gene

20   promoter   has   been   known   to   be   a   good   promoter   to   induce
high   expression   of   a   foreign   protein(Kim   et   al.,   J
Biochem(Tokyo).,   126(2);320-325,   1999)   and   is   a   good
candidate   to   express   a   large   scale   of   desired   foreign
proteins. Preferably, 5.5 kb to 10 kb is preferable as a

25   length.

"A second region" of herein, characterized by having
a length of 2.8 to 3.5 kb which is homologous to a DNA
sequence of bovine beta-casein gene, and comprising exon 5-
8 and intron 5-7 of bovine beta-casein gene.   3.0 kb to 3.2

30   kb is preferable as a length.

The first region is located upstream to the second region in the 5'-3' arrangement of beta-casein gene.

The term "homology", as used herein, means the degree of similarity between nucleic acid sequences of the first

5   region or a second region and the endogenous beta-casein gene sequences corresponding to these first and second regions. The sequences are homologous to each other when the sequences exhibit at least 90%, preferably at least 95% sequence identity.

10   The term "(multiple) cloning site", as used herein, means a nucleic acid sequence comprising at least two distinct nucleotide sequence specifically recognized and digested by restriction enzymes to permit insertion of nucleic acid sequence coding desired proteins.

15   A desired protein, which can inserted to the MCS on a vector of the present invention, may includes all proteins having medical or industrial application, such as hormones, cytokines, enzymes, coagulation factors, carrier proteins, receptors, regulatory proteins, structural proteins,

20   transcription factors, antibodies, antigens, etc.

Specific examples of the desired proteins include, but not limited to, thrombopoietin, human growth hormone, growth hormone releasing hormone, growth hormone releasing peptide, interferons, interferon receptors, colony-

25   stimulating factor, glucagon-like peptides, G-protein-coupled receptor, interleukins, interleukin receptors, enzymes, interleukin binding proteins, cytokine binding proteins, macrophage activating factor, macrophage peptide, B cell factor, T cell factor, protein A, suppressive factor

30   of allergy, cell necrosis glycoprotein, immunotoxin,

23

lymphotoxin, tumor necrosis factor, tumor inhibitory factor, transforming growth factor, alpha-1 antitrypsin, albumin, alpha-lactalbimin, apolipoprotein-E, erythroprotein, hyper-glycosylated erythroprotein, angiopoietins, hemoglobin, thrombin, thrombin receptor activating peptide, thrombomodulin , factor VII , factor VIIa, factor VIII, factor IX , factor XII , plasminogen activator, fibrin binding protein, urokinase, steptokinase, hirudin, protein C, C-reactive protein, renin inhibitor, collagenase inhibitor, superoxide dismutase, leptin, platelet derived growth hormone, epithelial growth factor, epidermal growth factor, angiostatin, angiotensin, osteogenic growth factor, osteogenesis stimulating protein, calcitonin, insulin, atriopeptin, cartilage inducing factor, elcatonin, connective tissue activator protein, tissue factor pathway inhibitor, follicle stimulating hormone, luteinizing hormone, FSH releasing hormone, nerve growth hormone, parathyroid hormone, relaxin, secretin, somatomedin, insulin-like growth factor, adrenocorticotrophic hormone, glucagon, cholecystokinin, pancreatic polypeptide, gastrin releasing peptide, corticotropin releasing factor, thyroid stimulating hormone, autotaxin, lactoferrin, myostatin, receptor, receptor antagonist, cell surface antigen, virus-derived vaccine antigen, monoclonal antibody, polyclonal antibody, and so on.

The term "selection marker", as used herein, is required for selecting transformed cell with the vector. The selection marker gene confers resistances to drugs, nutritional requirement and cytotoxic drugs, or induces selectable phenotype such as fluorescence and a color

deposit.   There is a positive selection marker and a
negative selection marker.   "Positive selection marker"
makes cell expressing positive selection markers to survive
against selective agent, so that be capable of conferring

5   positive selection characteristic for the cell expressing
that marker.   The positive selection marker includes, but
not   limited   to,   neomycin,   hygromycin,   histidinol
dehydrogenase, guanine phosphosribosyltransferase, and so
on.

10   The   vector   of   the   present   invention   comprises
negative   selection   marker   as   additional   component.
"Negative selection marker" removes cells with random-
integration, so that be capable of conferring negative
selection characteristic for the cell expressing that

15   marker.   The negative selection marker includes, but not
limited   to,   thymidine   kinase   (tk),   hypoxanthine
phosphoribosyl transferase (Hprt), cytosine deaminase,
diphtheria toxin (DT), and so on.   The negative selection
marker locates at the 5' terminus of a first DNA sequence

20   or the 3' terminus of the second DNA sequence. Among those
negative selection markers, tk and DT is used generally. In
the present invention, DT gene was utilized as a negative
selection marker.   Whereas tk requires treatment of
gancyclovir, DT does not require any other treatments. It

25   is also reported that tk-carrying cells treated with
gancyclovir show potent 'bystander effect' on co-culturing
with unmodified cells and gancyclovir suppress the growth
of cells (Yoshiyasu Kaneko et al., Cancer Letters, 96;105-110,
1995). In those aspects, DT is more preferable than tk.

30   The positive and negative selection markers of the

present invention have independent promoter and poly A
regions. The promoter includes, but not limited to, simian
virus 40 (SV40) promoter, mouse mammary tumor virus (MMTV)
promoter, human immunodeficiency virus-long terminal repeat

5   (HIV-LTR) promoter, moloney virus promoter, cytomegalovirus
(CMV) promoter, epstein-barr virus (EBV) promoter,
respiratory syncytial virus (RSV) promoter, RNA polymerase
II promoter, β-actin promoter, human hemoglobin promoter,
human muscle creatin promoter, and so on.

10   The nucleic acid coding the desired protein is
integrated to a beta-casein gene locus of cellular genomic
DNA in host cell by homologous recombination, and is
expressed in the cell instead of the endogenous beta-casein
protein.

15   To improve efficiency of homologous recombination
events, the bovine beta-casein gene targeting vectors of
the present invention have characteristics as follows.

The efficiency of gene integration of the nucleic
acid coding a desired protein into a beta-casein gene locus

20   of cellular genomic DNA has been shown to have relation
with the targeting vector system, especially with the
degree of similarity and length of nucleic acid sequences
of homologous regions (Scheerer et al., Mol Cell Biol.,
14(10)6663-6673, 1994; Thomas et al., Cell, 51(3);503-512,

25   1987; Hasty et al., Mol Cell Biol., 11(11);5586-5591, 1991;
Lu et al., Blood, 102(4);1531-1533, 2003). The present
invention maximized the efficiency by optimizing the
position and length of homologous region of the first and
second regions. In addition, to minimize positional

30   interference when homologous recombination events occurs,

the positive selection markers of the invented vectors were inserted into loci corresponding exon 3-4 and intron 2-4 regions of bovine beta-casein gene. In addition, in the present invention, multiple cloning sites (MCS) were
5   developed in the behind of "a first region" to insert foreign protein genes for ease. In addition, final vector scheme of the present invention comprising gene coding desired gene for gene targeting is different from the one of previous (SHEN Wei et al., Chinese Journal of
10 Biotechnology, 20(3);361-365, 2004). 3 of homology regions for homologous recombination events existed in vector of the previous report, because gene coding desired protein were inserted randomly into a first region of the vector (SHEN Wei et al., Chinese Journal of Biotechnology,
15 20(3);361-365, 2004). Whereas, only two homology regions for recombination event existed in vector of the present invention, because gene coding desired protein were inserted into MCS of vector. Accordingly, when the vector cassette of the present invention is used, construction of
20 the vector for gene targeting is simple and targeting efficiency of vector is much higher than that of previous one (SHEN Wei et al., Chinese Journal of Biotechnology, 20(3);361-365, 2004).

25   Indeed, the present vector system induced homologous recombination events even in the somatic cells that are transcriptionally silent, leading to the stable integration of the foreign protein gene into a beta-casein gene locus.

        In a preferred embodiment of this invention, pBCKI I
30 and pBCKI II vectors, and pBCKIDT I and pBCKIDT II vectors

27

harboring a negative selection marker were prepared as
targeting vectors.

pBCKI I vector, about 18.8kb in length, was derived
from pBluescript II SK(+) plasmid. The first region of
5   pBCKI I vector is about 10kb in length and it includes 8 kb
sequences harboring bovine beta-casein promoter, exon 1,
intron 1 and exon 2 of the bovine beta-casein gene. A
second region of pBCKI I vector is about 3.1 kb in length
and it includes exons 5-8, introns 5-7 and a portion of
10   intron 4 and 8 as shown in Fig. 2. The pBCKI I vector
includes a *neo* gene as a selection marker, SV40 early
promoter and polyA sequences. The pBCKI I vector includes
three restriction enzymes sites(Sac II, Not I and BamH I),
for the insertion of nucleic acid coding desired proteins
15   as shown in Fig. 1.

pBCK II vector, about 14.8kb in length, was derived
from pGEM7Zf(+) plasmid. The first region of pBCKI II is
about 6kb in length and it includes bovine beta-casein
promoter having a length of about 4kb and axon 1, intron 1
20   and exon 2 of the bovine beta-casein. A second region of
pBCKI II vector is about 3.1 kb in length and includes exons
5-8, introns 4-7 and a portion of intron 4 and 8 as shown
in Fig. 2. The pBCKI II vector includes a *neo* gene as a
selection marker gene, SV40 early promoter and polyA. The
25   pBCKI II vector includes three restriction enzymes sites(Sac
II, Not I and BamH I), for the insertion of nucleic acid
coding desired proteins as shown in Fig. 2.

The pBCKIDT I and pBCKIDT II vectors were developed as
inserting DT gene into Xho I and Sal I sites of the pBCKI I
30   and pBCKI II vectors.

28

The targeting vectors according the present invention can. be prepared using general DNA recombination techniques. Site-specific cleavage and ligation can be accomplished by using the enzymes known to the art.

5   In a specific embodiment, human thrombopoietin (TPO) gene was inserted into BCKI I and BCKI II(Fig 17, 18 and 19). The 1 kb human TPO cDNA fragment flanked by 0.3 kb fragment of bovine growth hormone (bGH) gene was constructed (Sohn' et al., DNA Cell Biol., 18(11);845-852, 1999). The bGH gene

10   was inserted to encode s stable messenger RNA.

Human thrombopoietin is one of the major hemopoietic regulators participating in series of megakaryocytopoiesis, that is, proliferation and differentiation of megakaryocytes inducing production of platelets. As a

15   primary physiological regulator of platelet production, it plays a pivotal role in promoting the proliferation and maturation of megakaryocytes. Severe neutropenia and thrombocytopenia are seen in patients undergoing aggressive chemotherapy and bone marrow transplantation for

20   hematologic malignancies and solid tumers (Lok et al., Stem Cells, 12(6);586-598, 1994; Kaushansky et al., Stem Cells, 15(1);97-102, 102-103, 1997; Kaushansky et al., Ann Intern Med., 126(9);731-733, 1997; Kaushansky et al., Leukemia, 11(3);426-427, 1997; Kaushansky et al., Annu Rev Med.,

25   48;1-11, 1997). It was confirmed that recombinant TPO has ability to ameliorate thrombocytopenia in animal models so that showed the potential use for therapeutic purpose. The safety and efficacy of TPO was approved in Phase I clinical trials (Fanucchi et al., N Engl J Med., 336(6);404-409,

30   1997; Basser et al., Lancet., 348(9037);1279-1281, 1996;

O'Malley et al., Blood, 88(9);3288-3298, 1996). It also appears that TPO could be used to enhance the recovery of platelet production in myelosuppressed patients undergoing cancer chemotherapy.

5   Particularly, *Escherichia coli* cells transformed with the pBCTPOKIDT II vectors were deposited with the international depositary authority as KCTC 10864BP at Korea collection for type cultures (KCTC, #52 Oun-dong Yuseong-gu, Daejeon, South Korea) on 17 October 2005.

10   Among the above two vectors, the pBCKI II vectors harboring the first region having a length of 6kb are more efficient than pBCKI I vectors in targeting to the beta-casein gene in genome. And, pBCKIDT II vectors harboring negative selection marker, DT gene, showed 4 to 5-fold

15   higher targeting efficiency than pBCKI II vectors. In the present invention, 36.6% bEF and 41.4% bESF cells were targeted with the pBCKITPOKIDT II vectors, and the targeting efficiency was 3.3-fold higher (41.4%/12.7%) than the that of previous targeting vectors, 12.7% in goat fetal

20   fibroblasts (SHEN Wei et al., Chinese Journal of Biotechnology, 20(3);361-365, 2004). Therefore, we confirmed that the vectors of the present invention are highly efficient bovine beta-casein gene targeting vectors.

25   In another aspect, the present invention relates to bovine somatic cell which is gene-targeted with the above vectors.

A cell targeted with a vector of the present invention is derived from eukaryotes, and can be primary,

30   secondary, or permanent cells. Preferably, the cells are

derived from cattle, sheep, goats, pigs, horses, rabbits, dogs, monkeys, and so on, but not limited to. Useful tissues capable of detaching or activating cells are, but not limited to, livers, kidneys, spleens, bones, marrows,

5   thymuses, hearts, muscles, lungs, brains, seminal glands, ovaries, islets, intestines, ears, skins, pancreatic tissues, prostate glands, bladders, embryos, immune systems, hematopoiesis systems, and so on. And cell types are, but not limited to, fibroblasts, epithelial cells, nerve cells,

10 embryonic cells, liver cells, ovarian follicle cells, and so on.

A transfection method of the vector includes any method of introducing a nucleic acid into the cell, and the transfection can be carried out using an appropriate

15 technique well known in this art. The transfection methods include, but not limited to, electroporation, calcium phosphate co-precipitation, retroviral infection, microinjection, DEAE-dextran, cationic liposome transfections and so on. When transfection, a linear form

20 of vectors, which is digested with a restriction enzymes, is more preferable than a circular form vectors, is exemplary.

In particular, a beta-casein targeting-vector inserted with a human thrombopoietin (hTPO) was introduced

25 into bovine embryonic fibroblasts (bEF) and bovine ear skin fibroblasts (bESF) using Lipofectamine™ 2000 reagent (Invitrogen). Cationic liposome interacts efficiently with negative charged DNA and the DNA-liposome complexes are associated with the cell membrane, thereby leading to DNA

30 internationalization. Insertion of the gene-targeting

vector into a specific gene was detected and confirmed by a long range PCR and a southern blot assay from the colonies survived after the treatment with antibiotics. Using the procedures, clones derived from bESF cells targeted with hTPO at the beta-casein gene were obtained. The number 81 cell clone was designated as BCTPOKIbESF81 and deposited with the Korean Collection for Type Cultures (KCTC) located in #52, Oun-dong, Yusong-ku, Taejon 305-333, Republic of Korea on Nov 10 2004, under the accession number KCTC-10720BP.

In another aspect, the present invention relates to embryo which is nuclear-transferred with the said bovine somatic cell.

The term, "Nuclear transfer" is a process that genetic material of the nuclear donor cells is transferred into the enucleated oocytes. The nuclear transfer technique makes it possible to produce genetically identical animal clones because genetic material of the same donor cell is transferred into the recipient cytoplast.

In another aspect, the present invention relates to a method for producing a bovine beta-casein gene-targeted somatic cell, which comprises the steps of (1) introducing the bovine beta-casein gene targeting vector into a bovine somatic cells; (2) occurring homologous events in the transfected somatic cell; and (3) selecting the bovine beta-casein gene-targeted somatic cell.

The bovine beta-casein gene targeting vectors of the present invention, more particularly, pBCKI I, pBCKI II,

pBCKIDT I, and pBCKIDT II were optimized to improve targeting efficiency. Accordingly, production efficiency of the bovine somatic cell which is bovine beta-casein gene-targeted with a gene coding a desired protein can be

5   improved by using the above vector.

In addition, the vector in the step (1) is introduced into cells in form of linearized or deleted form lacking plasmid vector backbone.

10   In another aspect, the present invention relates to a method to provide a method for preparing cloned transgenic cattle, which comprises the steps of (1) introducing the above vector into a bovine somatic cell; (2) occurring homologous recombination events in the bovine somatic cell;

15   (3) selecting the bovine beta-casein gene-targeted somatic cell with a desired gene by homologous recombination; (4) introducing the above gene-targeted cell into nuclear-removed bovine oocyte to produce nuclear-transferred embryo; and (5) implanting the above embryo into surrogates

20   to produce cloned transgenic cattle.

As referred above, the invention is proper to produce cloned gene-targeted cattle wherein the invented vectors are optimized for bovine beta-casein gene-targeting.

To remove the genetic materials of the oocytes,

25   various methods such as physical enucleation, chemical treatment and centrifugation with Cytochalasin B treatment are employed (Tatham et al., Hum Reprod., 11(7);1499-1503, 1996). In this invention, the physical enucleation method using a glass micropipette was used.

30   A gene-targeted somatic cell is introduced into a

33

nuclear-removed animal embryo by using the techniques such as cell fusion method and intracytoplasmic cell injection (ICCI), etc. The cell fusion method is simple and useful for large production of embryos. The ICCI method permits maximum exposure of a nucleus isolated from donor cells to the cytosol in recipient cytoplasts.

The fusion of somatic cell and enucleated oocyte is accomplished by changing charges on cell surface by electric pulse. It is convenient to use an electro-cell manipulator that the pulse length and voltage are easily controllable.

In another aspect, the present invention relates to a method for producing a desired protein from milk, which comprises the steps of introducing the vector according to the present invention into an animal somatic cell; selecting the cell targeted with a desired gene by homologous recombination; introducing the gene-targeted cell into a nuclear-removed animal embryo; implanting the embryo into a milk-producing animal to produce a transgenic animal; and producing milk from the transgenic animal.

The nuclear-transferred embryos are activated and developed in vitro to the blastocyst stage and then and then implanted to recipients.

The activation of cloned embryo induces reinitiation of cell cycle, which is temporarily quiescent, whereby the cleavage of embryo is possible. To activate a cloned embryo, the activation of cell cycle arrest factors, such as maturation-promoting factor (MPF), mitogen-activated protein (MAP) kinase, and so on, should be suppressed,

34

wherein for the suppression of the activation, the increase
of intracellular calcium ion in a cloned embryo is
necessary. The activation of cell can be accomplished by
the dramatic increase of calcium influx induced by electro-
5   stimulation or chemical treatment such as ionomycin, 6-
dimethylaminopurine (6-DMAP), and so on, wherein the above
methods can be used independently or together.   In the
present invention, the reconstructed embryos were treated
with   ionomycine+6-DMAP   and   developed   *in   vitro*   to   a
10   blastocyst stage.

As   a   result,   cloned   offspring   which   can   express
desired proteins during lactation period are generated.
Using the transgenic animals produced by the targeting
vector systems of the present invention, desired proteins
15   can be obtained in large quantity from the milk without
causing severe lethality during the embryonic or post-natal
development.

In another aspect, the present invention relates to a
20   method to obtain desired protein from milk of the cloned
transgenic cattle produced using above method.

The   desired   proteins   expressed   in   milk   can   be
purified using conventional methods such as salting out
(examples: ammonium sulfate precipitation; sodium phosphate
25   precipitation),   solvent   precipitation   (example: protein
fraction precipitation using acetone, ethanol and so on),
dialysis,   gel   filtration,   ion   exchange,   column
chromatography such as reverse column chromatography, ultra
filtration, combinations thereof, and so on (Maniatis et
30   al., Molecular Cloning: A Laboratory Manual, Cold Spring

35

Harbor Laboratory, Cold Spring Harbor, N.Y.(1982); Sambrook
et al., Molecular Cloning: A Laboratory Manual, 2d Ed.,
Cold Spring Harbor Laboratory Press(1989); Deutscher, M.,
Guide to Protein Purification Methods Enzymology, vol. 182.
5   Academic Press. Inc., San Diego, CA(1990))

    Hereinafter, the present invention will be described
in detail referring to the following examples.   However,
the examples according to the present invention can be
10 modified in other forms, and the scope of the present
invention is not limited to the following examples.

Example 1 - Bovine Gene targeting vector construction

15   **1-1 pBCKI I and pBCKI II vector construction**
    In this invention, we employed a bovine beta-casein
gene promoter locus which direct expression of foreign
therapeutic genes in the milk.
    The pBC 10 vector construct (FIG. 9) as previously
20 described (Sohn et al., J Biotechnol., 103(1);11-19, 2003)
contains a 8 kb promoter of 5'-flanking sequence, the
entire exon 1 and 2 kb intron 1 and 5'-UTR of exon 2 of the
bovine beta casein in a pBluescript II SK(+) plasmid
(Stratagene). The beta-casein gene region of the pBC 10
25 vector was used as a long arm (a first DNA region) of two
invented vector cassettes, pBCKI I and pBCKI II. As shown in
FIG. 10, a 10 kb beta-casein gene could be digested with
Sac I and Sac II restriction enzymes and it was used as a long
arm (a first DNA sequence) of the pBCKI I vector cassette
30 (FIG. 10 and 14). A 6 kb beta-casein promoter region

digested with Aat II and Sac II restriction enzymes, including 4 kb promoter, untranslated exon 1, 2 and intron 1 of the bovine beta-casein gene, was used as a long arm (a first DNA sequence) of BCKI II (FIG. 11 and 14).

5   The pBC3.1 vector (FIG. 12) was constructed to utilize as a short arm of the vector cassettes of the invention. The 3.2 kb DNA fragments of the bovine beta-casein gene sequence (4676 to 7898), containing exons 5, 6, 7, 8 and introns 5, 6, 7 and partial introns 4 and 8, was

10   amplified by PCR using bovine genomic DNA as the template. Sequences of the primers used for the PCR reaction are as follows:

SEQ ID No 1:

15   Forward primer: 5' - attcagtcgagtggaacataaactttcagcc - 3'
SEQ ID No 2:
Reverse primer: 5' - catatgtcgactgtgagattgtattttgact - 3'

Bold characters of the primer sequences depict that

20   some bases were changed to generate Xho I and Sal I restriction enzyme sites.

The PCR product (FIG. 12) was digested with Xho I and Sal I restriction enzymes and ligated into the Sal I site of pGEM-T (Promega). To generate enzyme sites to insert into

25   pBC10 vector, the 3.2 kb bovine beta-casein gene fragment in pGEM-T was digested with Hinc II and Sal I restriction enzymes. The resultant 3.1 kb fragment was ligated into Sal I site of pBluescript II SK(+) to generate the BC3.1 vector.

The neo gene fragment utilized as a selection marker

30   also was inserted into pBC10 vector. To obtain the neo gene

37

fragments including the SV40 *ori*, early promoter, the SV40
early splicing region and polyadenylation regions, a
pMAM*neo* vector (CLONTHECH) was digested with Bam
H Irestriction enzyme (FIG. 10). First, the resultant 2.7 kb

5   DNA fragment was ligated into Bam H I site of the
pBluescript II SK(+). Second, the 2 kb *neo* gene fragment in
the ligated vector was digested with Bgl II and Bam H I
restriction enzymes and then ligated into Bgl II site of the
pSP73 (Promega). Finally, the 2 kb *neo* gene fragment in

10 the pSP73 was digested with Bgl II and EcoR V restriction
enzymes and then re-ligated into the Bgl II and EcoR V sites
of the pBluescript II SK(+) harboring the 0.7 kb pMAMneo gene
fragment. These DNA digestion and ligation steps were
required for inserting the 2.7 kb *neo* gene fragment into

15 the pBC10 vector (FIG. 14).

As shown in FIG. 11, pBCKI I and pBCKI II vector
cassettes were accomplished by assembling p*neo*2.7 and
pBC3.1 gene fragments into the pBC10 vector. The *neo* gene
in pBluescript II SK(+) was digested with Bam H I and EcoR V

20 restriction enzymes and then ligated into Bam H I and EcoR V
sites of the pBC10 vector. The pBC3.1 vector was digested
with EcoR V and Sal I restriction enzymes and then ligated
into EcoR V and Sal I sites of the pBC10 vector including
the ligated pMAMneo gene fragment to generate the pBCKI I

25 vector cassette. The pBCKI I vector cassette was digested
with Aat II and Sal I restriction enzymes and ligated into
Aat II and Xho I sites of the pGEM-7Zf (Promega) to generate
the pBCKI II vector cassette. Therefore, the pBCKI I and
pBCKI II vector cassettes were constructed.

30

1-2 pBCKIDT I and pBCKIDT II vector construction

A negative selection marker, DT gene, was inserted into the above vectors (FIG. 3 and 16). As using pBCKIDT I and pBCKIDT II vectors harboring a negative selection marker,

5   non-targeted cell clones are reduced because most cell clones which have integrated the vectors at a random location are killed by toxicity of DT gene (FIG. 3 and 16). The DT gene utilized in the invention was isolated from pKO SelectDT vector (Lexicon Genetics, The Woodlands, Tex.).

10   Using a restriction enzyme, Rsr II, the Diphtheria toxin A chain (DT) gene with SV 40 Poly A and RNA polymerase II promoter was isolated. After klenow-filling the isolated DT gene, it was inserted into Hind II restriction sites of pBluescript II KS (+) (Stratagene), resulting pKS DT vector.

15   DT gene in the pKS DT vector was digested with Xho I and EcoR V and ligated into Xho I and Pvu II sites of pSP73 vectors (Promega), resulting pSP73 DT vector (FIG. 15). The DT gene in pSP73 DT vector was digested with Xho I and Sal I and then, inserted into Sal I site of pBCKI I vector cassette,

20   resulting pBCKIDT I vector. The pBCKIDT I vector was digested with Aat II and Sal I and ligated into Aat II and Xho I sites of pGEM-7Zf (+) (Promega), resulting pBCKIDT II vector cassette (FIG. 16).

The fidelity of the targeting-vector cassettes was

25   confirmed again by DNA sequencing and enzyme mapping on all regions of synthetic DNAs and all junctions of the ligated DNA fragments.

Example 2 - Construction of pBCTPOKI I and pBCTPOKI II

30

39

As shown in FIG. 17, 18 and 19, the 1 kb Human TPO cDNA plus 300bp bovine growth hormone was inserted pBCKI I, pBCKI II, and pBCKIDT II vector cassettes. The 1.3 kb foreign gene harboring Sac II and Not I sites was ligated into the
5 Sac II and Not I sites of the pBCKI I, pBCKI II, and pBCKIDT II vectors, respectively, thereby generating the targeting vectors, named as pBCTPOKI I, pBCTPOKI II, and pBCTPOKIDT II (FIG. 17, 18, and 19). Particularly, *Escherichia coli* cells transformed with the pBCTPOKIDT II vectors were deposited
10 with the Korean Collection for Type Cultures (KCTC) located in #52, Oun-dong, Yusong-ku, Taejon 305-333, Republic of Korea on Oct 17 2005, under the accession number KCTC 10864BP.

15 **Example 3 - Transfection of pBCTPOKI I, pBCTPOKI II, and pBCTPOKIDT II vector constructs into bovine embryonic fibroblasts (bEF) and bovine ear skin fibroblasts (bESF)**

3-1 Introduction of linearized vectors
20 The plasmid DNAs, pBCTPOKI I and pBCTPOKI II, were purified by using "QIAfilter Plasmid Midi kits" (Qiagen) and then closed circular DNAs were isolated by an equilibrium centrifugation in CsCl-ethidium bromide gradient. The isolated pBCTPOKI I and pBCTPOKI II plasmids
25 were linearized by digestion of Sal I(FIG. 20) and Aat II (FIG. 21) restriction enzymes, respectively, and then purified by ethanol precipitation. Finally, the concentrations of the purified DNAs were determined by using a spectrophotometer.
   The linearized pBCTPOKI I and pBCTPOKI II constructs
30 were introduced into the bEF and bESF at passage 2 or 3 by

using Lipofectamine 2000 reagent (Invitrogen). For bEF, we tested three different transfection reagent volumes such as 2, 4 and 10 μl, and two different DNA concentrations such as 2 and 4 μg. For bESF, 2, 4 and 10 μl transfection reagent volumes and 1, 2 and 4 μg DNA concentrations were tested. The somatic cells were exposed to the transfection reagent-DNA complexes for 24 h.

The somatic cells were cultured in Dulbecco Modified Eagle Medium (Gibco, Invitrogen corporation) supplemented with 10% FBS (Hyclone), 0.001% Gentamicine (Gibco, Invitrogen corporation) and 1% MEM Non-Essential Amino Acid (Gibco, Invitrogen corporation) and the culture medium was exchanged everyday. The cells were incubated at 37℃, 5% CO_2 in air. The volume of cell culture medium was different depending on the plate types used as shown below.

96 well	48 well	24 well	12 well	6 well	100 mm dish
0.2 ml	0.5 ml	1.0 ml	1.5 ml	3 ml	10 ml

The cells grown to confluence were treated with 1×Trypsin-EDTA (Gibco, Invitrogen corporation) solution at 37℃ for 3 min and then washed twice with Dulbecco's Phosphate-Buffered Saline (Gibco, Invitrogen corporation). Cell colonies detached by 1×Trypsin-EDTA treatment were dissociated by gentle pipetting and then transferred into the more wider plates for proliferation. The volumes of 1×Trypsin-EDTA solution used depends on cell culture plate types as shown below

96 well	48 well	24 well	12 well	6 well	100 mm dish

50 μl	100 μl	150 μl	200 μl	500 μl	1 ml

The procedure of the experiment is shown below:

Day 1	5.5×10^5 bEF cells and 3.6×10^5 bESF cells at passage 2 or 3 onto 6-well culture plates were transfected with the linearised BCTPOKI I and BCTPOKI II DNAs using Lipofectamine 2000 reagent (Invitrogen) in 2ml of OPTI-MEM IReduced Serum Medium (Gibco, Invitrogen corporation) according to the procedure recommended by the manufacturers.
Day 2	Transfected cells were split into two 100 mm culture dish and further cultured.
Day 3	0.8 -1.5 mg/ml G418 (Gibco, Invitrogen coporation) was added to the culture medium.
Day 4-14	Colonies of, approximately, 2-3 mm in diameter were picked and individually replated into wells of 96 well culture plates.

5   The cells grown until confluent in 96-well plates were transferred to 48 well culture plates and then were gradually expanded in 24 well, 12 well, 6 well and 100 mm culture plates.

     In 6 well plates, approximately half of the

10 transfected cells were picked for PCR reactions and the other was transferred into two wells of a 6 well culture plate.

     3-2 Introduction of linearized vectors after deleting

15 **plasmid vectors**

     The plasmid vector regions of pBCTPOKI II and pBCTPOKIDT II vector constructs were deleted by digesting

with restriction enzymes, Aat II and Cla I, and then, transfected into bESF and bEF cells as referred above (FIG. 22 and 23).

5 **Example 4 - Identification of the transfected cell clones by PCR analysis**

To screen transgenic cell lines, G418 resistant-colony cells were analyzed by PCR. Genomic DNAs were
10 purified from half of the cells in 6 well plates using "AccuPrep Genomic DNA Extraction Kit" (Bioneer) and PCR was carried out using "AccuPower PCR Premix" (Bioneer). Primer sets for human TPO cDNA and thermal cycling conditions are shown below:
15

SEQ ID No 3
Forward primer : GGA GCT GAC TGA ATT GCT CCT CGT
SEQ ID No 4
Reverse primer : CCT GAC GCA GAG GGT GGA CCC TCC
20

1 cylcle of	94 ℃	2 min
30 cycles of	94 ℃	1 min
	65 ℃	30 sec
	72 ℃	45 sec
1 cycle of	72 ℃	10 min

As results of the PCR, most cell clones resistant to G418 identified as transgenic dell (FIG. 17).

Example 5 - Long-range PCR to detect targeted cell clones

Long-range PCR was employed to determine the gene-targeted cell clones among transgenic cell clones. Genomic DNAs from each transgenic cell clone were purified using "AccuPrep Genomic DNA Extraction Kit" (Bioneer) or exposed by 3-4 cycles of freezing in liquid nitrogen and thawing in boiling water. Long-range PCR was performed using "AccuPower HL PCR Premix" (Bioneer).

As shown in FIG 5, the 5' primer was designed to bind at the 3' end of neo gene within the transfected vector constructs and the 3' primer to bind at the intron 8 of bovine beta casein gene which exists in the endogenous region, not in the vector construct. The gene targeted cell clones showed represented 4 kb PCR products on 1% agarose gel. The sequences of a primer set and thermal cycling conditions are shown below:

SEQ ID No 5 Forward primer: 5'-ccacacaggcatagagtgtctgc- 3'

SEQ ID No 6 Reverse primer: 5'-ccacagaattgactgcgactgg- 3'

1 cylcle of	92 ℃	2 min
35 cycles of	92 ℃	20 sec
	65 ℃	45 sec
	68 ℃	3 min
1 cycle of	68 ℃	10 min

Bovine-beta casein gene targeting efficiency was compared in examples 3-1 and 3-2.

In example 3-1, two cell clones were identified to be

gene-targeted (FIG. 28).

As shown in examples 3-1, 41 single colonies were isolated after the transfection of the pBCTPOKI Ivector into the somatic cells. Among these cells, 38 colonies (93%) were identified to be transgenic, but no targeted colonies (0%) were detected. 31 single colonies were isolated after transfection of the pBCTPOKI II vector into the somatic cells. Among these, 29 colonies(94%) were identified as transgenic cells, and 2 colonies (7%) were identified to be gene targeted with BCTPOKI II vector at the endogenous bovine beta-casein gene locus in genome (Table 1 & FIG. 28).

Table 1. Transfection and targeting efficiencies of BCTPOKI Iand BCTPOKI II vectors

	Cell types	BCTPOKI I		BCTPOKI II	
No. of analyzed colonies	bEF	23	41	2	31
	bESF	18		29	
No. of transgenic colonies	bEF	22 (96%)	38 (93%)	1 (50%)	29 (94%)
	bESF	16 (89%)		28 (97%)	
No. of targeted colonies	bEF	0 (0%)	0 (0%)	0	2 (7%)
	bESF	0 (0%)		2	

As shown in examples 3-2, pBCTPOKI II and pBCTPOKIDT II vectors were transfected into bEF and bESF cells and targeted cell clones were confirmed using long-range PCR analysis. Here, for small volume of DNA samples, secondary PCR analysis was performed (FIG. 27 and 29).

Thermal cycling conditions of secondary PCR reaction

are same as those of example 5. The targeted cell clones
showed represented 3.4 kb PCR products on 1% agarose gel.
The sequences of a primer set of secondary PCR reaction are
shown below:

5   SEQ ID No 8

Forward primer: 5'-ttcactgcattctagttgtggtttgtcca-3'

SEQ ID No 9

Reverse primer: 5'-tctaggaccaaacatcggcttactt-3'.

As shown in FIG. 29 A, the number 5, 30, 17, 18, 20,
10 21, and 26 bESF cell clones transfected with pBCTPOKIDT II
vectors were identified to be targeted. B shows that the
number 16 bESF cell clone transfected with pBCTPOKI II vector
is targeted. C is the results of long-range PCR analyses of
wild-type bovine genomic DNA used as a negative control (-)
15 and pneoBC3.7 vector used as a positive control (+).

pBCTPOKI II and pBCTPOKIDT II vectors were transfected
into bEF and bESF cells and targeting efficiencies were
compared. 18.2% (10/55) bEF cell clones transfected with
pBCTPOKI II vectors and 41.4% (12/29) bEF cell clones
20 transfected with pBCTPOKIDT II vectors were identified to be
gene-targeted, showing targeting efficiency by the
pBCTPOKIDT II vectors is 2.3-fold (41.4%/18.2%) higher than
that by the pBCTPOKI II vectors. And, 5.7% (12/212) bESF cell
clones transfected with pBCTPOKI II vectors and 36.6%
25 (63/172) bESF cell clones transfected with pBCTPOKIDT II
vectors were identified to be gene-targeted, showing
targeting efficiency by the pBCTPOKIDT II vectors is 6.4-fold
(36.6%/5.7%) higher than that by the pBCTPOKI II vectors. The
average targeting efficiency of the pBCTPOKIDT II vectors in
30 both cell types was 4.5-fold (37.3%/8.3%) higher than that

of pBCTPOKI Ⅱ vectors (Table 2), indicating the pBCKIDT Ⅱ
vector cassettes are highly efficient bovine beta-casein
gene-targeting vectors. In addition, the targeting
efficiency by pBCTPOKIDT Ⅱ vectors in bEF cells was 3.3-fold
5   higher (41.4%/12.7%) than the that by previous targeting
vectors, in goat fetal fibroblasts (SHEN Wei et al.,
Chinese Journal of Biotechnology, 20(3);361-365, 2004),
indicating the invented vectors are highly efficient gene-
targeting vectors.

10

Table 2. Transfection and targeting efficiencies of
pBCTPOKI Ⅱ and pBCTPOKIDT Ⅱ vectors

	Cell types	No. cell clones Analyzed	NO. cell clones targeted	%	%
pBCTPOKI Ⅱ	bEF	55	10	18.2%	8.3%
	bESF	212	12	5.7%	
pBCTPOKIDT Ⅱ	bEF	29	12	41.4%	37.3%
	bESF	172	63 ·	36.6%	

15   **Example 6 - Southern blot analysis to reconfirm targeted
cell clones**

Using vectors described example 1, bovine beta-casein
gene targeting was accomplished, following methods referred
20   to example 3. As results, targeting efficiency was various,
depending on types of vectors and transfected cells.
Here, both cell clones targeted with pBCTPOKI Ⅱ
vectors, using methods of example 3-1, were re-analyzed by

47

the southern blotting.

Two clones identified as gene-targeted by the PCR reactions were analyzed again by the southern blotting. The two cell clones were transferred to two 100 mm culture
5   dishes respectively. Cells were harvested from one of the two dishes, and at least 10 micrograms DNA from each clone was extracted and digested with EcoR1 for 16hr at 37℃. EcoR I-digested DNA was separated by electrophoresis through a 0.75% agarose gel at 50V in 1 x TAE buffer for 16hr. The
10 DNA was transferred onto nylon membranes positively charged (Boehringer Mannheim) and hybridized with the probe targeting human TPO cDNA in the vector constructs (see FIG. 30). The probes for southern blotting were prepared with primers of SEQ ID NO3 and 4 following the guidelines
15 (Roche). The PCR reaction was carried out using "PCR DIG labeling mix" (Roche) and "Taq DNA polymerase" (QIAGEN). Thermal cycling conditions were as below:

1 cylcle of	94℃	3 min
	94℃	45 sec
30 cycles of	52℃	30 sec
	72℃	1 min
1 cycle of	72℃	10 min

20   The gene targeting of the previously identified two clones were confirmed again by the southern blotting (FIG. 31). The two targeted cell clones are number 81 and number 89 cell lines.

The number 81 cell clone was designated as
25 BCTPOKIbESF81 and deposited with the Korean Collection for

48

Type Cultures (KCTC) located in #52, Oun-dong, Yusong-ku,
Taejon 305-333, Republic of Korea on Nov 10 2004, under the
accession number KCTC-10720BP.

5   **Example 7 - Preparation of bovine somatic cells**

        Experiments were conducted according to the Animal
Care and Use Committee guidelines of the National Livestock
Research Institute of Korea. Bovine embryonic fibroblasts
10   (bEF) were isolated from fetus at Day 45 of gestation of
the Holstein cow which produce over 12,000kg milk a year
and prepared as described previously (Koo et al., Biol
Reprod., 63(4);986-992, 2000). The head of the fetus was
removed using iris scissors, and soft tissues such as liver
15   and intestine were also discarded by scooping out with two
watchmarker's forceps. Bovine ear skin fibroblasts (bESF)
were isolated from the ear skin of a 2-years-old Holstein
cow which produces over 12,000kg milk a year. After twice
washing with PBS (Gibco BRL), the bEF and bESF were minced
20   with a surgical blade on a 100 mm culture dish. These
procedures were performed at room temperature. The minced
tissues were incubated in 10 ml of 0.05% (w/v) trypsin/0.53
mM EDTA solution in an incubator at 38.5℃ for 30 min.
Trypsin was inactivated by adding an equal volume of cell
25   culture medium supplemented with 10% FBS. The cell culture
medium was composed of Dulbecco modified Eagle medium
supplemented with 10% FBS, 1000 units of penicillin and
1000 µg/ml of streptomycin (Gibco BRL). After vigorous
pipetting, the supernatant was centrifuged at 150 X g for 5
30   min. The cells were suspended and adjusted to a final

concentration of 2×10^6 cells/ml, and then were cultured in

10ml culture medium at 37°C in 5% CO_2 in air in 175-cm^2

tissue culture flasks (Nunc, Roskilde, Denmark) until

confluent. The bEF and bESF cells were frozen down in cold

5 Dulbecco's Phosphate-Buffered Saline solution supplemented

with 20% FBS and 10% Dimethyl sulphoxide and frozen at -70°C

for 16hr. The cells were stored in liquid nitrogen until

transfection experiment.

To obtain the gene-targeted cell lines, DNA-

10 transfected cells should survive for a long period of

culture in vitro without morphological modification and

apoptosis. In this invention, BCTPOKI I and BCTPOKI II vectors

were introduced into two types of somatic cells: bEF and

bESF. It was reported that ovine post-natal fibroblasts

15 persist through longer periods maintaining a relatively

stable chromosomal karyotype in culture than in ovine fetal

fibroblasts (Williams et al., Mol Reprod Dev., 66(2);115-

125, 2003). In this invention, the bESF sustained much

longer normal morphologies in in vitro culture than bEF

20 (Table 2).

Table 2. Cell survival rate on passage 4 and passage 8

Cell type	No. of colonies		Survival rate
	P4	P8	
bEF	149	9	6%
bESF	304	51	17%

51 (17%) of 304 bESF colonies on passage 4 were

25 expanded by passage 8, showing normal morphologies. However,

50

in case of bEF cells, only 6% clones were cultured by passage 8. In this experiment, we obtained two gene-targeted clones from bESF cells.

5   **Example 8 - Freezing and thawing of frozen gene-targeted cells**

When the gene-targeted cells were grown to confluent in two 100mm culture dishes, cells of one plate were
10   subjected to Southern blot analysis. And half of the other plate cells were further expanded and the other half stored in liquid nitrogen after freezing at -70°C for 16hr. The procedures of cell sub-culturing and storing were repeated to obtain a lot of cells for use as donor cells. The cell
15   freezing medium consisted of DMEM supplemented with 20% FBS and 10% dimethyl sulphoxide (DMSO).

After thawing one vial of frozen gene-targeted cells as soon as possible, the solution was transferred into 15 ml of tube containing 9 ml of cell culture medium and
20   centrifuged 1000 rpm for 3 min. The cell pellet was resuspended in 3 ml culture medium, plated on 6-well culture plate containing 3 ml of culture medium, and then cultured at 37°C in 5% CO_2 in air prior to nuclear transfer.

25   **Example 9 - Nuclear Transfer**

Bovine oocytes obtained from slaughterhouse ovaries were cultured in the maturation medium at 38.57°C in 5% CO_2 in humidified air. The maturation medium consisted of TCM-
30   199 (Sigma Chemical Co.) with Eagle salts and L-glutamine

51

supplemented with 10% (v/v) FBS (Gibco BRL, Grand Island,
NY), 1 μg/ml estradiol, 1 μg/ml FSH-P (Schering-Plough
Animal Health Corp., Kenilworth, NJ), and 25 mM $NaHCO_3$.
After *in vitro* maturation, the oocytes were transferred to
5   500 μl of TL-Hepes supplemented with 0.1% hyaluronidase and
then cumulus of the oocytes were removed by mechanical
pipetting. The zonae pellucida of denuded oocytes were
partially dissected using a fine glass needle (Tsunoda et
al., J Exp Zool., 240(1);119-125, 1986). Oocytes
10   manipulations such as enucleation and cell injection were
performed using a micromanipulator equipped with an
inverted microscope (Leitz, Ernst Leitz Wetzlar GmbH,
Germany). The medium used for manipulation was TL-Hepes
containing 7.5 μg/ml cytochalasin B. A first polar body and
15   partial cytoplasm presumptively containing metaphase II
chromosomes were removed together by using a micropipette
with an inner diameter of 20 μm. Single gene-targeted cells
were individually transferred to the perivitelline space of
the recipient cytoplast. The cell-cytoplast complexes were
20   equilibrated in a 50 μl drop of cell fusion medium for 10-
20 sec and then transferred to a fusion chamber with two
electrodes 1mm apart overlaid with cell fusion medium. The
cell fusion medium consisted of 0.3 M mannitol, 0.5 mM
Hepes, 0.01% BSA, 0.1 mM $CaCl_2$, and 0.1 mM $MgCl_2$. The cell-
25   cytoplast complexes were induced to fuse with a single
pulse of direct current of 1.6 kV/cm for 20 μsec by an
Electro Cell Manipulator 2001 (BTX, San Diego, CA). These
procedures were performed at room temperature.
Reconstructed embryos without visible somatic cells were
30   determined as fused eggs 1 h after the fusion pulse. At 4 h

after electrofusion, the fused eggs were activated with 5 μM ionomycin for 5 min, followed by treatment with 2.5 mM 6-dimethyl-aminopurine in CR1aa medium (Rosenkrans et al., Biol Reprod., 49(3);459-462, 1993) supplemented with 10%

5   FBS for 3.5 h at 38.5°C in 5% CO$_2$ in air.

Example 10 - Culture of reconstructed embryos

The reconstructed embryos were cultured in CR1aa

10   medium supplemented with 1 mM glutamine and 1 X Eagle essential amino acids solution (Gibco BRL). After culture for 3 days, cleaved embryos were further cultured in each well of a 4-well culture plate containing 750 μl CR1aa on a mouse embryonic fibroblasts monolayer (with 10% FBS) for 4

15   days at 38.5°C in 5% CO$_2$ in air (Park et al., Anim Reprod Sci., 59(1-2);13-22, 2000). After 7 days of culture, blastocyst formation was observed.

Example 11 - PCR analysis of cloned embryos

20

Each embryo was transferred into 20 μl of lysis buffer, which consisted of 50 mM KCl, 1.5 mM MgCl$_2$, 10 mM Tris-HCl pH8.5. 0.5% Nonidet P40, 0.5% Tween and 400 μg/ml Proteinase K, and incubated at 65°C for 30 min. Proteinase

25   K was inactivated at 95°C for 10 min (McCreath et al., Nature, 405(6790);1066-1069, 2000). For the each lysed embryo, the first PCR was carried out with primers of SEQ IN Nos 3 and 4 using the "AccuPower PCR Premix" (Bioneer) and then the nested PCR was performed using 1 μl of the

30   first PCR product. The primers of IN Nos 3 and 7 were used

53

and thermal cycling conditions were as follows:

Nested PCR

  SEQ ID No 7

5   Reverse primer: 5'-gagacggacctgtccagaaagctg-3'

1 cylcle of	94 ℃	2 min
	94 ℃	1 min
30 cycles of	65 ℃	30 sec
	72 ℃	45 sec
1 cycle of	72 ℃	10 min

It was analyzed by PCR at various different developmental stages whether the cloned embryos were 10 transgenic or not (Table 3).

Table 3. PCR analysis of reconstructed embryos at various different developmental stages

	Developmental stages							
	1 cell	2 cell	4 cell	8 cell	16 cell	morula	blastocyst	Total
No. of analyzed embryos	3	3	6	11	2	3	5	33
No. of transgenic embryos	3	3	6	11	2	3	5	33

15

As a result, 33 (100%) of 33 cloned embryos were transgenic. The results indicate that the present invention could produce the gene-targeted cloned animals by using the gene-targeted somatic cells.

Figure 32 shows the PCR analysis result of nuclear-transferred embryos from cell clone 81 (FIG. 23)

Example 12 - Long-range PCR analysis of a fetal membrane derived from a cloned fetus.

Reconstructed embryos at the blastocyst stages were transferred to recipients by a non-surgical method. At 36 days of gestation, a fetus and fetal membrane were flushed from the uterus of the cow non-surgically using a Foley catheter (Agtech, Manhatan, KS). The extracted fetus and fetal membrane derived from the fetus were cultured *in vitro* as described in example 7. The long-range PCR result, showing 4kb band, confirmed that cultured cells derived from the fetal membrane were accurately targeted into a beta-casein gene in genome with the vector of the present invention (FIG. 33). The procedures of genomic DNA extraction and long-range PCR analysis are the same as described in example 5.

Industrial Applicability

As described herein, the transgenic cattle, which are prepared by implanting a nuclear-transferred embryo introduced with a cell targeted with bovine beta-casein gene targeting vector, can produce a large scale of biomedically or biotechnically valuable proteins in their milk without lethality during embryonic or post-natal developmental stage by unregulated expression of the foreign proteins.
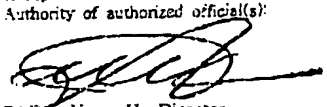
BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT
OF MICROORGANISMS FOR THE PURPOSE OF PATENT PROCEDURE

INTERNATIONAL FORM

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT

issued pursuant to Rule 7.1

TO : HAN, Yong-Mahn
Hanul Apt. 110-305,
Sinsong-dong, Yuseong-gu, Daejeon 305-345,
Republic of Korea

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR: BCTPOKI6ESF81 (primary cell line)	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: **KCTC 10720BP**

II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION
The microorganism identified under I above was accompanied by: [x] a scientific description [   ] a proposed taxonomic designation (Mark with a cross where applicable)

III. RECEIPT AND ACCEPTANCE
This International Depositary Authority accepts the microorganism identified under I above, which was received by it on **Nov 10 2004.**

IV. RECEIPT OF REQUEST FOR CONVERSION
The microorganism identified under I above was received by this International Depositary Authority on   and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on

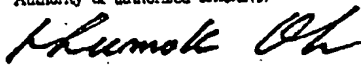| V. INTERNATIONAL DEPOSITARY AUTHORITY	
Name: Korean Collection for Type Cultures Address: Korea Research Institute of Bioscience and Biotechnology (KRIBB) #52, Oun-dong, Yuseong-ku. Taejon 305-333, Republic of Korea	Signature(s) of person(s) having the power to represent the International Depositary Authority of authorized official(s): PARK, Yong-Ha Director Date: Nov 15 2004

56

INTERNATIONAL FORM

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT

issued pursuant to Rule 7.1

TO : HAN, Yong- Mahn
Hanul Apt. 110-315,
Sinseong-dong, Yuseong-gu, Daejeon 305-707,
Republic of Korea

I. IDENTIFICATION OF THE MICROORGANISM

Identification reference given by the DEPOSITOR:	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY:
Escherichia coli DH5@/pBCTPOKIDT II	KCTC 10864BP

II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I above was accompanied by:
[x] a scientific description
[   ] a proposed taxonomic designation
(Mark with a cross where applicable)

III. RECEIPT AND ACCEPTANCE

This International Depositary Authority accepts the microorganism identified under I above, which was received by it on **October 17, 2005.**

IV. RECEIPT OF REQUEST FOR CONVERSION

The microorganism identified under I above was received by this International Depositary Authority on   and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on

V. INTERNATIONAL DEPOSITARY AUTHORITY

Name: Korean Collection for Type Cultures	Signature(s) of person(s) having the power to represent the International Depositary Authority of authorized official(s):
Address. Korea Research Institute of Bioscience and Biotechnology (KRIBB) #52, Oun-dong, Yusong-ku, Taejon 305-333, Republic of Korea	OH, Hee-Mock, Director Date: October 21, 2005